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Protein kinase A-dependent activation of PDE4 (cAMP-specific cyclic nucleotide phosphodiesterase) in cultured bovine vascular smooth muscle cells

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Abstract

Incubation of cultured bovine vascular smooth muscle cells (VSMC) with forskolin increased cAMP as measured by an increase in cAMP-dependent protein kinase (PKA) activation (PKA ratio). Forskolin also produced a concentration- and time-dependent increase in activity (3–5-fold within 15 min) of a PDE4 (cAMP-specific cyclic nucleotide phosphodiesterase). The increase in PDE4 activity was not affected by cycloheximide and thus not likely due to increased synthesis of the enzyme. Activation, which was preserved during partial purification of the enzyme by chromatography on Sephacryl S-200 and MonoQ, was most likely due to a covalent modification. Incubation of cell homogenates with the catalytic subunit of PKA (PKA_c) induced a ~5-fold activation of PDE4 with a time course similar to that in intact cells after forskolin addition. The forskolin-mediated activation was reversed during incubation of homogenates at room temperature for two hours. Addition of PKA_c resulted in rapid reactivation of PDE4. These data are consistent with the hypothesis that rapid, reversible activation of PDE4 in cultured VSMC is mediated by PKA.

Keywords: Phosphodiesterase; Smooth muscle cells; cAMP dependent protein kinase; Rolipram; Forskolin

1. Introduction

Intracellular concentrations of cAMP are regulated both by alteration of rates of synthesis by adenylyl cyclases and of degradation by cyclic nucleotide phosphodiesterases (PDEs). Seven PDE families, PDE1-7, have been identified and characterized [1–7].

PDE4 isoenzymes have a high affinity for cAMP and are relatively sensitive to inhibition by rolipram and Ro-20-1724. PDE4s are products of at least four distinct genes in humans and rats [8,9]. The corresponding isoenzymes, named PDE4A-D, are ex-

Abbreviations: AEC, 3-amino-9-ethyl carbazole; DMEM, Dulbecco's modified Eagle medium; EC, effective concentration; FSH, follicle stimulating hormone; NOS, nitric oxide synthetase; PDE, phosphodiesterase; PKA, protein kinase A; PKA_c, protein kinase A, catalytic subunit; PKI, protein kinase inhibitor; PP, protein phosphatase; SM, smooth muscle; TSH, thyroid stimulating hormone; VSM, vascular smooth muscle; VSMC, vascular smooth muscle cell(s)

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pressed in many tissues, but in different amounts and proportions. Whereas PDE4C and PDE4D mRNAs have not been detected in peripheral blood cells or lymphocyte-derived cell lines, respectively, PDE4A and PDE4B mRNAs are found in most tissues [10]. Different PDE4s can be generated from the same gene, as is the case for one PDE4 gene (the so called 'ratPDE3/IVd') from which three PDE4 mRNAs can be produced in brain, Sertoli cells and FRTL-5 cells, a thyroid-derived cell line. Of PDE4 isoforms synthesized from these mRNAs, one was phosphorylated and activated by PKA in vitro, whereas the two others were constitutively active [11]. Rapid activation of PDE4 in FRTL-5 cells via cAMP-dependent phosphorylation was recently reported [12].

In vascular smooth muscle (VSM), representatives of at least four of the seven cyclic nucleotide PDE families are present: PDE1, PDE3, PDE4 and PDE5 [13]. Specific PDE inhibitors, effective in intact cells, have been used to evaluate the role of different PDEs in regulation of VSM relaxation, which is promoted by agents that increase cAMP [14]. Although both PDE3 and PDE4 are present in VSM and specific PDE3 inhibitors are potent vasodilators, PDE4 inhibitors alone produce little or no relaxation [15]. PDE4 inhibitors do, however, potentiate effects of PDE3 inhibitors [13]. Inhibitors of PDE3, but not PDE4, relax rat aorta denuded of endothelium [13]. In rat aorta preparations with intact endothelium, the effects of PDE4 inhibitors are enhanced by PDE3 inhibitors or by nitrovasodilators and other agents that increase cGMP; they are blocked by the nitric oxide synthase (NOS) inhibitor L-*N*-methyl-arginine [13]. These results suggest that in these VSM preparations when PDE3 is inhibited by PDE3 inhibitors or endogenously produced cGMP, increased cAMP is hydrolyzed primarily by PDE4. In rat aortic smooth muscle, stimulation of guanylyl cyclase also results in increased cAMP, probably via cGMP-inhibition of PDE3 [16]. In spite of the effect of rolipram on intact vessels in vitro, no obvious cardiovascular effects of rolipram in healthy human volunteers and patients were found [17]. Inhibition of PDE4, as well as of PDE3, had a pronounced relaxing effect in bronchial smooth muscle in vitro. For this reason, combined PDE3-PDE4 inhibitors such as Zardaverine have been considered as possible bronchodilating drugs [18–22].

We report here for the first time that a PDE4 in cultured VSMC is rapidly activated by forskolin and by PKA_c in cell homogenates, findings consistent with the hypothesis that activation in intact VSMC is mediated by PKA-catalyzed phosphorylation.

2. Material and methods

2.1. Cell culture

VSMC from bovine aorta were cultured as described by Crossman et al. [23] with minor modifications. Bovine aortas, obtained from a local slaughterhouse, were opened longitudinally, the internal surfaces were scraped with a scalpel to remove endothelium, and explants ($\sim 2 \times 2$ mm), cut from the media, were placed in 25-cm^2 tissue culture flasks, (usually ~ 10/flask), where they were allowed to adhere for 3 h in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal calf serum, penicillin 100 IU/ml and streptomycin 100 μg/ml. Additional DMEM (5 ml) was added and the flasks were incubated at 37°C in air with 5% CO_2 and 100% humidity. Media were changed every 3–4 days; confluent cultures were obtained in about 2 weeks. Cells were harvested after incubation with trypsin (0.25% trypsin, 1 mM EDTA (Gibco), 50 μl/cm^2, 10 min, 37°C) and distributed to 6-well cell culture plates (Costar), where cultures became confluent in ~ 1 week.

For immunostaining of α-smooth muscle (SM) actin, cells were grown in plastic chambers on slides (Nunc Chamber Slides, 4 wells). Cells were fixed with ethanol/5% acetic acid at -20°C for 20 min, incubated with monoclonal antibodies against α-SM actin (Sigma Diagnostics), stained with peroxidase-coupled secondary antibody and 3-amino-9-ethyl carbazole (AEC) chromogen and mounted in glycerol-gelatin. In experiments reported here, cells, usually confluent cultures, were immunopositive for α-SM actin.

2.2. Preparation of supernatants from VSMC incubated with forskolin

Confluent cultures were washed twice with DMEM without serum, then incubated at 37°C in DMEM with indicated additions and 5% CO_2. Experiments

were usually performed in the presence of 100 μM N^{ω}-nitro-L-arginine to inhibit any NOS that might be induced.

Cells were harvested by scraping with a plastic cellscraper (Costar) in 200–1000 μl of homogenizing buffer (50 mM Tris, pH 7.3, 1 mM EDTA, 5 mM $MgCl_2$, 0.03% of the non-ionic detergent $C_{13}E_{12}$, 10% glycerol, antipain (20 μg/ml), leupeptin (10 μg/ml), pepstatin (10 μg/ml), 50 mM phenyl phosphate, 25 mM NaF) and transferred to a cooled glass homogenizer (Konte). They were homogenized (10 strokes) and the homogenate was centrifuged (4°C, $12\,000 \times g$, 15 min) to obtain the supernatants used for experiments reported here.

2.3. PDE assay

PDE activity was assayed by a modification of a published [24] procedure. Samples of supernatants were incubated for 8 min at 30°C in a total volume of 300 μl of 50 mM Hepes, pH 7.5, 0.1 mM EDTA, 8.3 mM $MgCl_2$, 0.5 μM ^{3}H-cAMP (NEN, ~250 cpm/pmol cAMP). Hydrolysis of substrate did not exceed 20% and PDE activity was proportional to enzyme concentration. PDE4 activity was calculated as activity in absence of rolipram minus activity in presence of 10 μM rolipram (Schering). Activity is reported as pmol cAMP hydrolyzed/min per mg supernatant protein (mean of duplicate assays). The proportion of PDE4 was estimated at 40–60% of total PDE activity.

Protein was assayed by the method of Bradford [25] with albumin (2–10 μg) as standard.

2.4. PKA assay

PKA activity was determined according to the method of Egan et al [26]. Samples were incubated for 10 min at 30°C in a total volume of 60 μl of 20 mM MOPS, pH 7.0, 16 mM Mg-acetate, 125 μM ATP, γ-^{32}P-ATP (~5 Ci/mmol, ~10^6 cpm/sample), 4 mM DTE, 60 μM Kemptide, in the presence or absence of 16 μM cAMP (which induces maximal activation of PKA). The amount of Kemptide phosphorylated in the presence or absence of cAMP was determined by scintillation counting and the ratio was calculated. The degree of activation of PKA was expressed as this ratio.

2.5. Activation of PDE4 by PKA_c in vitro

VSMC supernatants (200 μl in homogenisation buffer, ~40–80 pmol/min total PDE activity) were incubated at 30°C for 15 min with bovine PKA_c (Sigma P2645), 100 U/ml, in a final volume of 900 μl containing 50 mM Hepes, pH 7.5, 40 μM ATP, 5 mM $MgCl_2$, 5 mM EDTA, 150 μM EGTA, 1 mM dithiothreitol, 0.5 μM cAMP, antipain (20 μg/ml), leupeptin (10 μg/ml) and pepstatin (10 μg/ml).

2.6. Sephacryl S-200 and MonoQ chromatography

VSMC supernatant (600 μl, 60–120 pmol/min PDE4 activity) was applied to a column (0.9×45 cm) of Sephacryl S-200 (Pharmacia) equilibrated and eluted with 50 mM Tris, pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 0.03% $C_{13}E_{12}$, 10% glycerol and 100 mM NaCl at a flow rate of 3 ml/h. 1-ml fractions were collected for assay of PDE activity.

VSMC supernatant (600 μl, 60–120 pmol/min PDE4 activity) was diluted to 2 ml in 50 mM Tris, pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 0.03% $C_{13}E_{12}$, 10% glycerol, antipain (20 μg/ml), leupeptin (10 μg/ml), pepstatin (10 μg/ml), 50 mM phenyl phosphate, 25 mM NaF. A sample (2 ml) was applied to a MonoQ HR5/5 column (Pharmacia) previously equilibrated in dilution buffer and eluted with a linear gradient of 0–500 mM NaCl (1 ml/min, 40 ml). 1-ml fractions were collected for assay of PDE activity.

3. Results

3.1. Effects of forskolin on PKA and PDE4 activity in VSMC

Stimulation of VSMC with forskolin resulted in a concentration-dependent increase in cAMP, estimated by an increase in the PKA ratio (Fig. 1), and in PDE4 activity (Fig. 2). Stimulation of the PKA ratio was maximal at 60 μM forskolin (Fig. 1).

Incubation of VSMC with 100 μM forskolin resulted in a rapid increase in PDE4 activity in VSMC supernatants (Fig. 3). PDE4 activity was increased within the first few minutes of incubation with forskolin, was close to maximal within 10–15 min

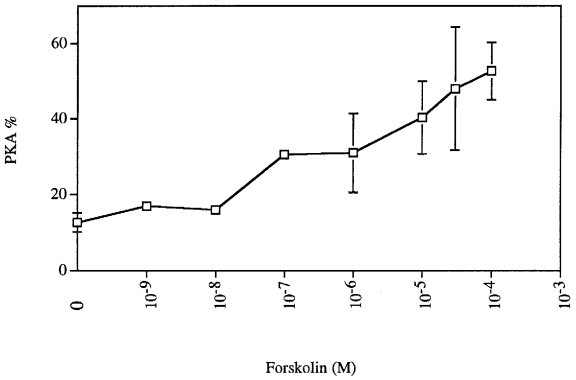

Fig. 1. Concentration dependence of forskolin stimulated PKA activity. VSMC (10^6 cells) were incubated for 10 min with different concentrations of forskolin. The cells were homogenized and centrifuged, and PKA activity ratios in the supernatants were assayed in duplicate as described in Section 2. Datapoints represent the mean ± S.E.M. of data from 3 experiments.

and then remained elevated for at least ~60 min (Fig. 3). Incubation of the cells with cycloheximide (100 μg/ml) for 60 min before addition of forskolin did not significantly affect the increase in PDE4 activity measured after 10 min with forskolin, suggesting that the increased activity was not related to enzyme synthesis (data not shown).

Forskolin-induced activation of PDE4 was maintained during Sephacryl S-200 chromatography of VSMC supernatants from control and forskolin stimulated VSMC. Total PDE4 activity in fractions from

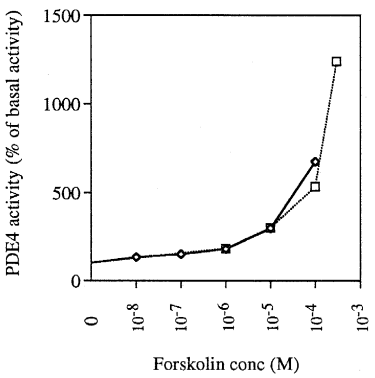

Fig. 2. Effect of forskolin concentration on activation of PDE4 in VSMC. VSMC were incubated in DMEM for 10 min with the indicated concentration of forskolin and harvested, and specific PDE4 activity was measured in VSMC supernatants. Results of two experiments are presented as % of activity in unstimulated cells at $t = 0$.

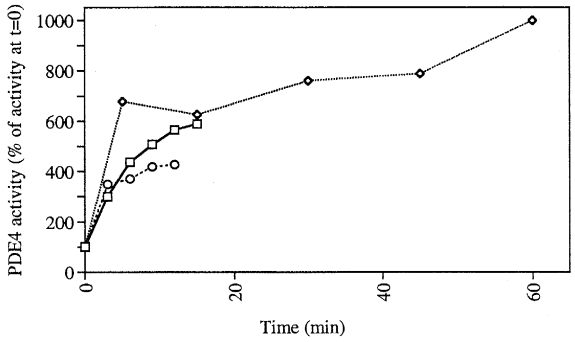

Fig. 3. Time course of activation of PDE4 by forskolin in VSMC. VSMC were incubated in DMEM with 100 μM forskolin for indicated times, harvested and specific PDE4 activity was measured in VSMC supernatants as described. Data from three experiments are presented as % of activity of unstimulated cells.

forskolin-stimulated cells was 3.7-fold higher than in corresponding fractions from control cells (Fig. 4, lower insert). After MonoQ chromatography, PDE4

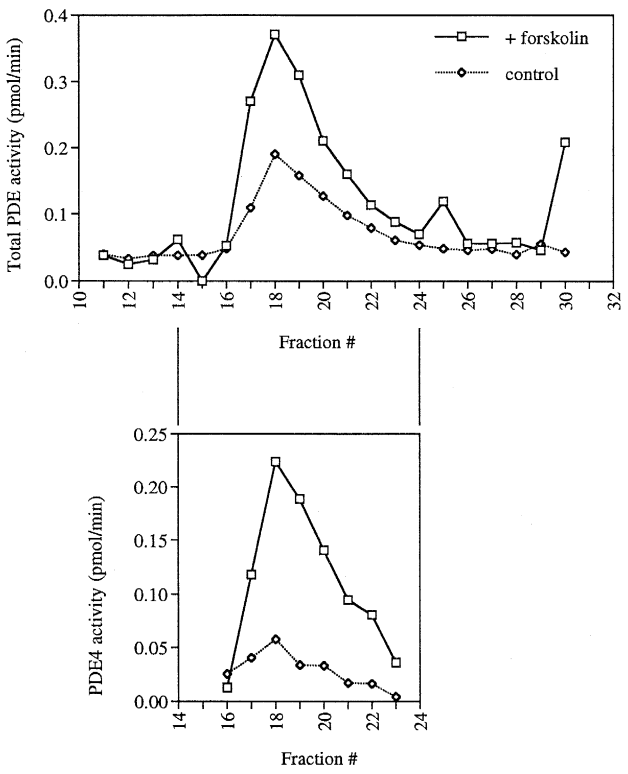

Fig. 4. Sephacryl S-200 chromatography of supernatants from control and forskolin stimulated cells. VSMC were incubated in DMEM for 10 min without or with 100 μM forskolin before homogenization. Supernatants were subjected to gel filtration on Sephacryl S-200. Total PDE activity (top) and PDE4 activity (bottom) was measured in indicated fractions.

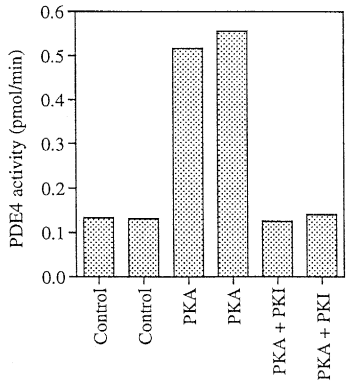

Fig. 5. Activation of PDE4 by PKA_c in vitro. Duplicate samples from pooled VSMC supernatants were incubated for 15 min at 30°C in presence of ATP without or with PKA_c and PKI as indicated before measurement of PDE4 activity.

activity in samples from stimulated cells was 1.3–1.7 times that in samples from control cells (data not shown). Taken together, these results suggest that a PKA-dependent phosphorylation is most likely responsible for forskolin-induced activation of PDE4 in VSMC.

3.2. In vitro activation of PDE4 by PKA_c

Incubation of VSMC supernatant with PKA_c and ATP resulted in a ~5-fold rise in PDE4 activity (Fig. 5), with a time-course (Fig. 6) similar to that observed in intact VSMC incubated with forskolin (Fig. 3). Activation was completely blocked by protein kinase inhibitor (PKI) (Fig. 5).

During incubation of homogenates from

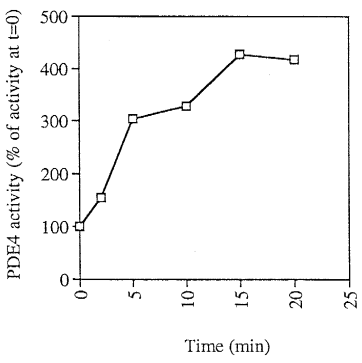

Fig. 6. Time course of activation of PDE4 by PKA_c in vitro. Samples from pooled VSMC supernatants were incubated at 30°C with PKA_c and ATP and PDE4 activity measured at indicated times.

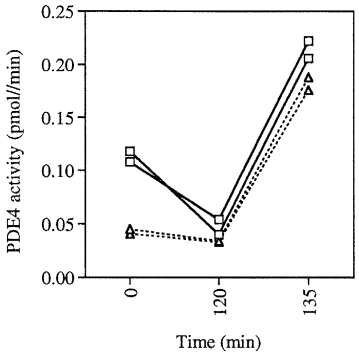

Fig. 7. Reversal of forskolin activation of PDE4 and reactivation with PKA_c. Duplicate VSMC cultures were incubated for 10 min with (□) or without (△) 100 μM forskolin. The cells were homogenized and PDE4 activity measured in supernatant fractions ($t = 0$). Samples of supernatants were incubated at room temperature for 120 min and some were then further incubated at 30°C for 15 min with PKA_c under activation conditions before assay of PDE4 activity.

forskolin-stimulated VSMC at room temperature for 2 h, PDE4 activity decreased almost to control values (Fig. 7). Incubation with PKA_c and ATP for 15 min induced a 5-fold increase in of PDE4 activity, indicating that inactivation was not due to protein degradation or other irreversible processes. The reduction in activity was not significantly increased by addition of protein phosphatase 2A (PP2A) to the sample. The PP2A-specific inhibitors okadaic acid (0.25 μM) and microcystin LR (10 μM) only slightly reduced the inactivation (data not shown), suggesting that PDE4 was inactivated by endogenous phosphatase(s) other than PP2A.

4. Discussion

It has recently been reported that a PDE4 isoenzyme in thyroid-derived FRTL-5 cells incubated with thyroid-stimulating hormone (TSH) was rapidly activated by PKA-dependent phosphorylation [12]. Hormones that elevate cAMP are also known to increase PDE4 mRNA and enzyme activity by increasing gene transcription, e.g., in Sertoli cells and in FRTL-5 cells in response to follicle-stimulating hormone (FSH) [27] and TSH [12], respectively. Similarly, dibutyryl-cAMP and forskolin increased PDE4 mRNA in skeletal muscle myoblasts [28].

Here we present for the first time evidence for

rapid and reversible PKA-dependent activation of a PDE4 in VSMC. As is the case in Sertoli cells and FRTL5 cells, a direct phosphorylation of VSMC PDE4 is most likely, although it can not be completely ruled out that PKA mediates its effect through other kinase(s). Activation, which occurred within less than 15 min in response to the adenylyl cyclase activator forskolin, was not inhibited by cycloheximide, suggesting that it was not due to synthesis of new enzyme. It was also preserved during partial purification, suggesting that it reflected a stable, covalent modification. In vivo, forskolin induced activation of PKA, and, in vitro, incubation with PKA_c activated PDE4 with the same time course as that in intact cells incubated with forskolin, supporting the idea that rapid activation in VSMC is in fact mediated by PKA.

The functional significance of the acute regulation of PDE4 by PKA in VSMC is not understood. The increase in PDE activity in response to increased cAMP could play a role in heterologous desensitization to hormone stimulation of adenylyl cyclase. Also, it has been shown in a cell-free system that, under conditions of rapid cAMP turnover, half-maximal PKA activation occured at a much lower cAMP concentration than required at slow turnover [29]. It is possible that such a mechanism could be operative in stimulated VSMC, where in the presence of increased PDE activity (and perhaps increased turnover of cAMP) PKA could be activated by relatively small increases in cAMP.

Variations in cAMP and consequently in PKA activity are known to influence both cell proliferation and contraction in VSMC. On the one hand, cAMP is involved in regulation of proliferation of certain cells, and inhibition of both PDE3 and PDE4 antagonizes proliferation of cultured rat [30] and porcine [31] aortic smooth muscle cells, human [32] and rat [33] T-lymphocytes, and rat mesangial cells [34]. Salbutamol, a β-receptor agonist, blocks proliferation of cultured human airway smooth muscle cells [35]. On the other hand, in VSM, hormones and agents which increase cAMP also lower calcium (probably at least in part by cAMP-mediated stimulation of reuptake into the sarcoplasmatic reticulum) and promote relaxation [14]. Activation of PDEs by cAMP-elevating agents might modulate cAMP-mediated relaxation or reduce the duration of the relaxation.

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